Notch1 but Not Notch2 Is Essential for Generating Hematopoietic Stem Cells from Endothelial Cells

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Summary

Hematopoietic stem cells (HSCs) are thought to arise in the aorta-gonad-mesonephros (AGM) region of embryo proper, although HSC activity can be detected in yolk sac (YS) and paraaortic splanchnopleura (P-Sp) when transplanted in newborn mice. We examined the role of Notch signaling in embryonic hematopoiesis. The activity of colony-forming cells in the YS from Notch1$^{-/-}$ embryos was comparable to that of wild-type embryos. However, in vitro and in vivo definitive hematopoietic activities from YS and P-Sp were severely impaired in Notch1$^{-/-}$ embryos. The population representing hemogenic endothelial cells, however, did not decrease. In contrast, *Notch2*$^{-/-}$ embryos showed no hematopoietic deficiency. These data indicate that Notch1, but not Notch2, is essential for generating hematopoietic stem cells from endothelial cells.

Introduction

During mouse embryogenesis, hematopoiesis begins in the yolk sac (YS) blood island at embryonic day (E)7.5 and then shifts to the fetal liver after E11.5 and later to spleen and bone marrow. Hematopoiesis before the formation of the fetal liver is known as primitive hematopoiesis, and consists of mainly nucleated erythrocytes with embryonic-type globin. Lymphopoietic cells and hematopoietic progenitors giving rise to adult-type blood cells are detected in the paraaortic splanchnopleura (P-Sp) region of mouse embryo at E7.5–9.5 (Godin et al., 1995; Delassus and Cumano, 1996). Long-term repopulating hematopoietic stem cells (LTR-HSCs) that can reconstitute adult mice originate from the intraembryonic aorta, gonads, and mesonephros (AGM) region at E10.5–11.5 (Muller et al., 1994; Cumano et al., 1996; Medvinsky and Dzierzak, 1996). Although a lack of the *Runx1* (Okuda et al., 1996) or *GATA-2* (Tsai et al., 1994) gene is known to be lethal to the mouse embryos due to defects in definitive hematopoiesis but not in primitive hematopoiesis, the mechanism of definitive hematopoiesis regulation is largely unknown.

The *Notch* gene family is highly conserved from the nematode through the vertebrates (Artavanis-Tsakonas et al., 1999) and plays an important role in determining cell fate in multiple systems. In vertebrates, four members of this gene family, *Notch1* through *Notch4*, have been isolated (Ellisen et al., 1991; Weinmaster et al., 1992; Lardelli et al., 1994; Uyttendaele et al., 1996). Among the Notch family proteins, Notch1 and Notch2 have been shown to be expressed in the hematopoietic progenitor cells (Milner et al., 1994; Bigas et al., 1998). Infection of mouse bone marrow cells with a retrovirus containing a constitutive active form of Notch1 resulted in the establishment of immortalized, cytokine-dependent cell lines that generated progeny with either lymphoid or myeloid characteristics both in vitro and in vivo (Varnum-Finney et al., 2000). Furthermore, Notch ligands, Jagged1, Jagged2, and Delta1, can inhibit differentiation of hematopoietic progenitor cells (Varnum-Finney et al., 1998; Jones et al., 1998; Carlesso et al., 1999; Han et al., 2000). Finally, Jagged1 was recently shown to be a novel growth factor for hematopoietic stem cells (Karanu et al., 2000). When these findings are taken together, the Notch signal is likely to prevent hematopoietic progenitor cells from differentiating. However, in a recent report on the phenotype of *Notch1*-conditional knockout mice, in which Notch1 was disrupted after birth, only T cell developmental deficiency was observed, while nonlymphoid hematopoiesis was apparently normal (Radtke et al., 1999).

Recently, Notch1 and Notch4 have been shown to be essential for vascular morphogenesis, especially in angiogenesis (Krebs et al., 2000). The development of hematopoietic cells is closely related to angiogenesis, indicating the existence of hemangioblasts and hemogenic endothelial cells. There is evidence supporting the existence of hemangioblast in YS (Choi et al., 1998). Within the embryo proper, the origin of the definitive hematopoietic cells was pinpointed to the dorsal aorta, where budding of hematopoietic cells from endothelial cells was observed histologically (de Bruijn et al., 2000). In mice heterozygous for the knockin allele of Runx1 with lacZ, lacZ staining was detected in the endothelial cells of the dorsal aorta and in associated hematopoietic clusters (North et al., 1999). In Ly-6A (Sca-1) green fluorescence protein (GFP) transgenic mice, GFP was expressed in all functional HSCs in the midgestation aorta and specifically localized to the endothelial layer lining the wall of the dorsal aorta (de Bruijn et al., 2002). These results imply the existence of hemogenic endothelial cells and indicate that definitive hematopoietic cells are of endothelial cell origin.

In this study, to identify the role of Notch signaling in HSC development we performed P-Sp organ culture using *Notch1*$^{-/-}$ and *Notch2*$^{-/-}$ embryos. Hematopoietic cell development together with angiogenesis was severely impaired in P-Sp of the *Notch1*$^{-/-}$ but not of the *Notch2*$^{-/-}$ embryo. Although colony forming cell (CFC)

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activity in YS was maintained in the *Notch1*$^{-/-}$ mice, HSC activity was undetectable in both P-Sp and YS of the *Notch1*$^{-/-}$ embryo. Since the number of surface marker-defined hemogenic endothelial cells did not decrease in either YS or P-Sp, we propose that Notch1 plays an essential role in preparing endothelial cells for hematogenicity.

Results

Notch Family Proteins Are Differentially Expressed during Hematopoietic Development

In order to determine the role of Notch signaling in development of hematopoietic cells, we performed immunostaining of the AGM-derived cells with antibodies recognizing respective Notch family members. Previously, it was reported that among the Notch family members, only Notch1 and Notch4 are expressed in the endothelial cells in the AGM region (Villa et al., 2001) and that Notch1 and Notch2 are expressed in hematopoietic stem cells (Milner et al., 1994; Bigas et al., 1998). Thus, we first investigated the expression of Notch1, Notch2, and Notch4 in the endothelial and hematopoietic cells in the P-Sp region at E9.5 before and after the culture. Before the culture, Flk1(+) endothelial cells expressed Notch1 and Notch4 (Figure 1A). After a 7 day culture, round-shaped CD45(+) hematopoietic cells were produced that expressed Notch1 and Notch2 but not Notch4 (Figure 1B). Endothelial cells at this point continued to express Notch1 and Notch4 (data not shown). Since 1, 1′-dioctadecyl-3, 3, 3′, 3′-tetramethylindo-carbocyanine perchlorate (DiI-Ac-LDL), which is specifically incorporated by the endothelial cells, was incorporated in the CD45(+) hematopoietic cells at this stage, it was indicated that these cells were progenies of the endothelial cells (data not shown) (Hara et al., 1999). Essentially the same expression patterns were observed for Notch1, Notch2, and Notch4 in the cells prepared from E10.5 AGM before and after the culture (data not shown). We next stained the whole embryo sections for Notch1 to evaluate its in situ expression around P-Sp and AGM from E9.5–E11.5 embryos. The aortic wall was stained for Notch1 in these sections throughout these stages. Notably, in the E11.5 AGM sections, we found specific staining of a monolayer representing the CD31(+) aortic endothelial cells and budding cells from the ventral side of the aorta, which are presumably hematopoietic cells dividing from endothelial cells (Figure 1C).

Both Hematopoiesis and Angiogenesis Are Impaired in the P-Sp Culture from Notch1-Deficient Mice

Several genes essential for hematopoiesis (for example *SCL*, *GATA-2*, and *Runx1*) are shown to be expressed in both hematopoietic and endothelial cells (Orkin and Zon, 2002). Since the expression pattern of Notch1 belongs to this category, we hypothesized that Notch1 might also be essential for hematopoietic development. In mice, Notch1 deficiency arrests development at or shortly before E9.5, and is embryonic lethal at or soon after E10.5 because of several defects including angiogenesis. There is, however, no information about the hematopoietic development (Swiatek et al., 1994; Conlon et al., 1995; Krebs et al., 2000). E9.5 Notch1$^{-/-}$ embryos were similar or only slightly reduced in size compared to wild-type embryos at the same stage, whereas E10.5 Notch1$^{-/-}$ embryos were markedly reduced in size and had obvious morphological abnormalities, as described previously (Swiatek et al., 1994; Conlon et al., 1995). We therefore performed in vitro P-Sp organ culture of the *Notch1*$^{-/-}$ embryo at E9.5 to analyze vasculo-angiogenesis and hematopoiesis. In this culture, PECAM-1(+) endothelial cells formed a sheet-like structure (vascular bed, vb) and a network structure (vascular network, vn), which reflect vasculogenesis and angiogenesis, respectively (Takakura et al., 1998). Although the *Notch1*$^{+/-}$ explant culture was not different from that of wild-type (data not shown), the *Notch1*$^{-/-}$ explant culture showed markedly impaired vn formation but little defect in vb formation (Figure 2A). This pattern, which demonstrates impaired angiogenesis, was similar to the result previously shown in the *Runx1*$^{-/-}$ embryo vasculature. In this culture system, it was also shown that hematopoietic cell development was severely impaired in the *Notch1*$^{-/-}$ embryo (Figure 2B). Furthermore, the CFC assay using hematopoietic cells from the cultured *Notch1*$^{-/-}$ P-Sp showed that there were few CFCs and no mixed colony forming cells (Figure 2C). These results indicate that Notch1 is indispensable for angiogenesis and hematopoietic development from the P-Sp region.

CFCs Were Preserved in the YS of *Notch1*$^{-/-}$ Mice

There were obviously reduced but visible red blood cells in the *Notch1*$^{-/-}$ embryo, which most likely represent primitive hematopoiesis. Therefore, to examine to what extent the primitive hematopoiesis was preserved, we investigated the CFC activity in YS of the *Notch1*$^{-/-}$ embryo. Surprisingly, the numbers and sizes of blood cell colonies derived from the cells prepared from *Notch1*$^{-/-}$ YS were very similar to those from wild-type YS, showing only a slight decrease in the total colony number (Figure 2D). The number of mixed colonies was not reduced (Figure 2D). Since we confirmed the expression of βH1-globin in all colonies individually examined, they were thought to have originated from primitive hematopoietic progenitors (data not shown) (Palis et al., 1999).

The Hemogenic Program Is Impaired in the P-Sp of the *Notch1*$^{-/-}$ Embryo

To explore the mechanism of impaired hematopoietic development in the *Notch1*$^{-/-}$ P-Sp, we used a semi-quantitative RT-PCR method to compare the expression levels of several transcription factors that were shown to be essential for various aspects of hematopoiesis (Figure 3A). The mRNA levels for *SCL*, Lmo2, *GATA-1*, *GATA-2*, and *Runx1* were reduced in *Notch1*$^{-/-}$ P-Sp. These results suggested that the cell-autonomous hemogenic program is impaired in the P-Sp of the *Notch1*$^{-/-}$ embryo.

To elucidate the mechanism of impaired angiogenesis, we analyzed the expression levels of several regulatory cytokines for angiogenesis and their receptors. The mRNA levels for all the genes studied, however, were unchanged between the *Notch1*$^{-/-}$ P-Sp and the wild-type P-Sp (Figure 3B). Thus, there was no direct evidence suggesting the association of Notch1 signaling

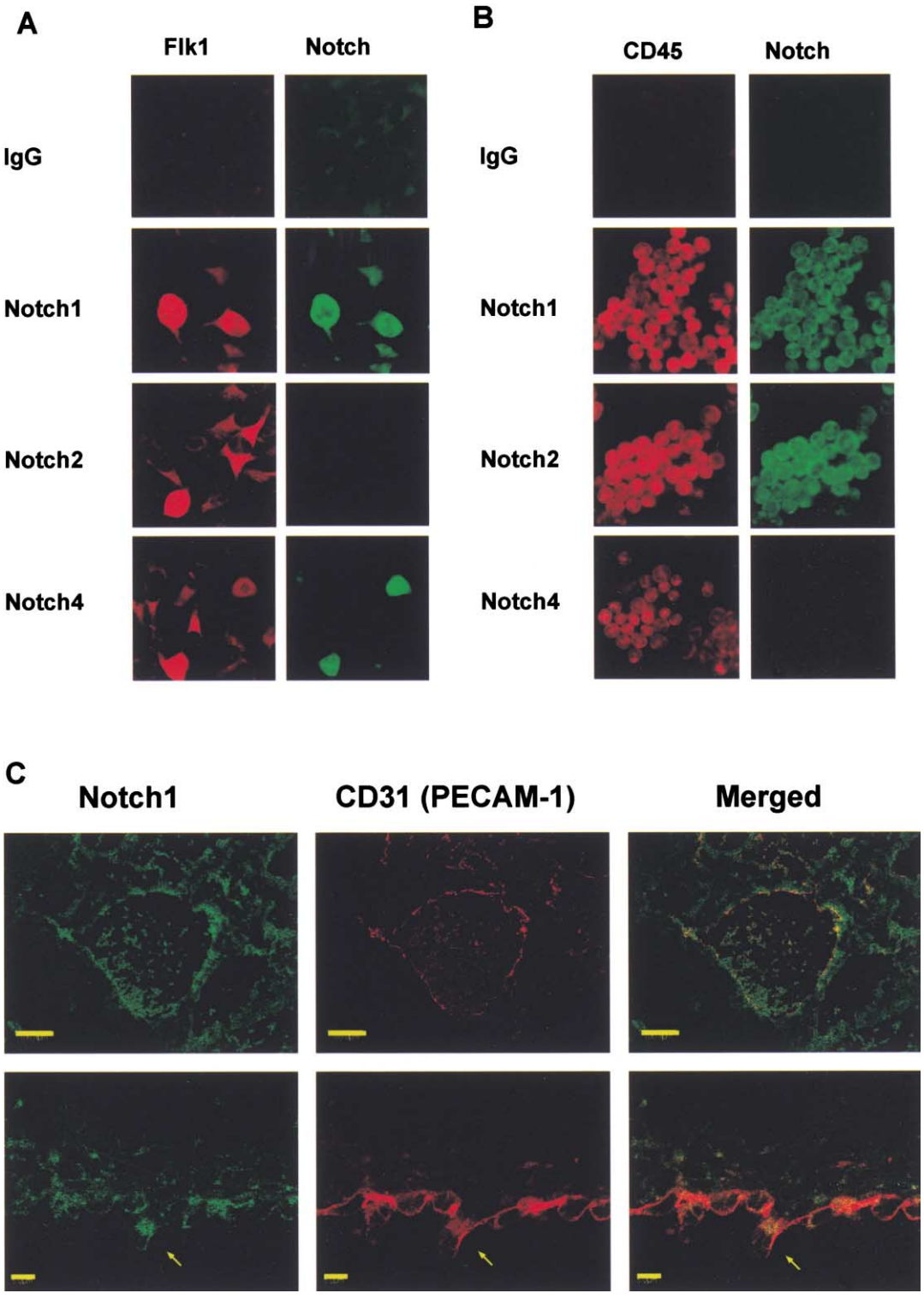

Figure 1. Expression of Notch Family Proteins in the Cells Derived from P-Sp Before and After the Culture and in the AGM Region from Whole Embryo Sections

Single cells derived from E 9.5 P-Sp were cultured on OP-9 stromal cells and the development of hematopoietic cells was observed. Immunostaining using control IgG, anti-Notch1, Notch2, and Notch4 antibodies was performed for (A) the Flk1 (+) endothelial cells at culture day 1, and (B) the CD45(+) hematopoietic cells at culture day 7. Magnification, ×200. (C) Immunohistochemical staining of frozen section of the E 11.5 AGM region of a mouse embryo with anti-Notch1 (green) and PECAM-1 (red). Both PECAM-1(+) endothelial cells and hematopoietic clusters expressed Notch1. Arrowheads indicate the hematopoietic clusters. Upper panel: lower magnification (×20). Upper right and lower left are located to the ventral and dorsal side of the embryo, respectively. Scale bar, 100 μm. Lower panel: higher magnification (×100). Scale bar, 5 μm.

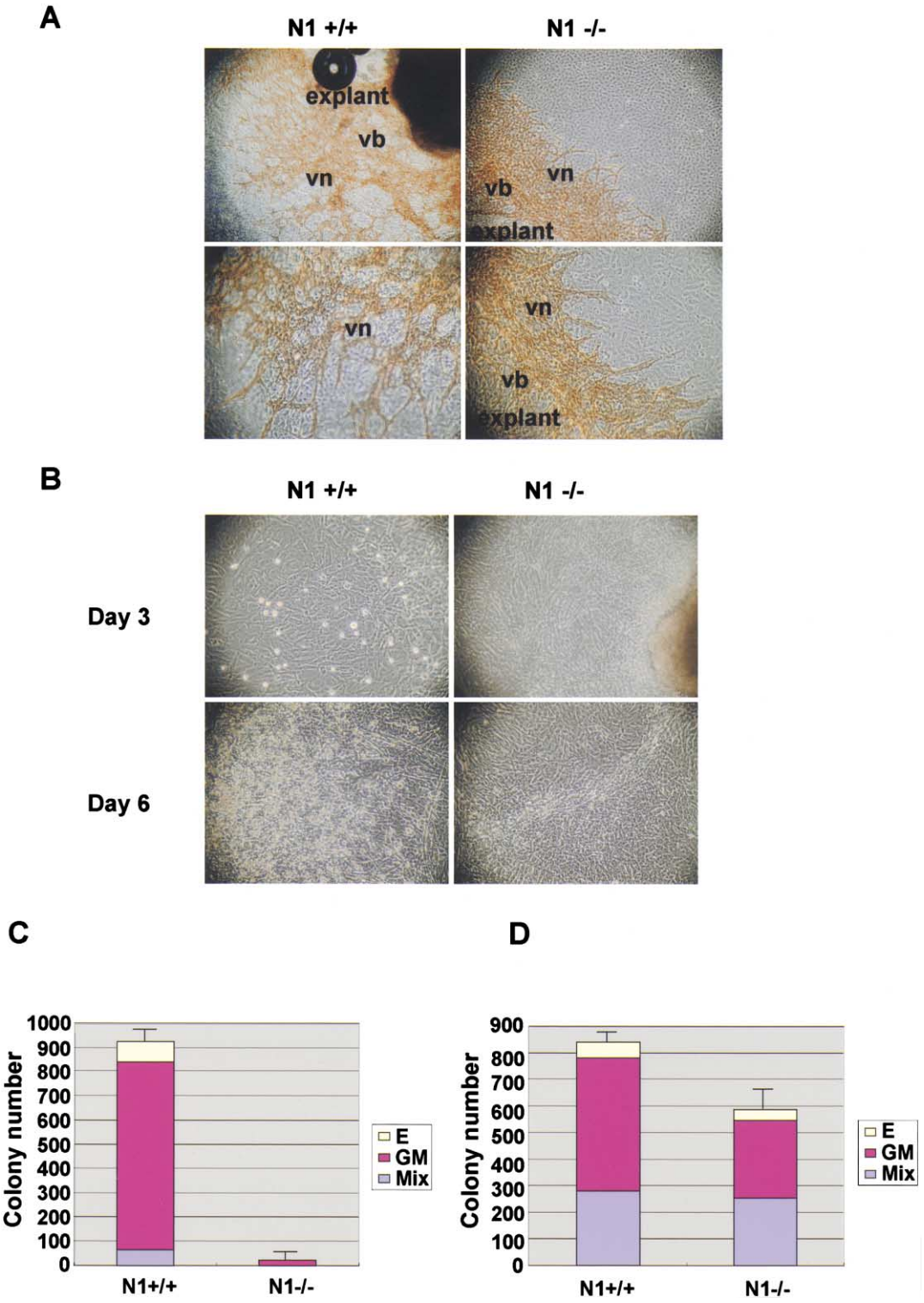

Figure 2. Angiogenesis and Hematopoiesis Were Impaired in the P-Sp Region in the *Notch1* Mutant Embryo, but CFC Activity of YS Was Maintained

P-Sp explants from *Notch1*$^{+/+}$ and *Notch1*$^{-/-}$ embryo at E 9.5 were cultured on OP-9 cells. (A) Endothelial cells at culture day 7 were immunostained by an anti-PECAM-1 antibody. Upper panel, lower magnification (×100); lower panel, higher magnification (×200). vb, vascular bed; vn, vascular network. (B) Hematopoietic cells emerged at day 3 and at day 6 from a *Notch1*$^{+/+}$ P-Sp whereas no hematopoietic cells were observed in the culture of *Notch1*$^{-/-}$ P-Sp. Magnification, ×200. CFC activity of the cells harvested after day 7 from the P-Sp organ culture (colonies per cultured cells from a P-Sp) (C) and of the cells freshly prepared from YS (colonies per 1 × 10^5 cells) (D) from *Notch1*$^{+/+}$ (N1+/+) and *Notch1*$^{-/-}$ (N1−/−) embryos. The results show the mean values of triplicate wells with standard deviations in one representative experiment from three independent experiments. E, erythroid colony; GM, granulocyte-macrophage colony; mix, mixed colony.

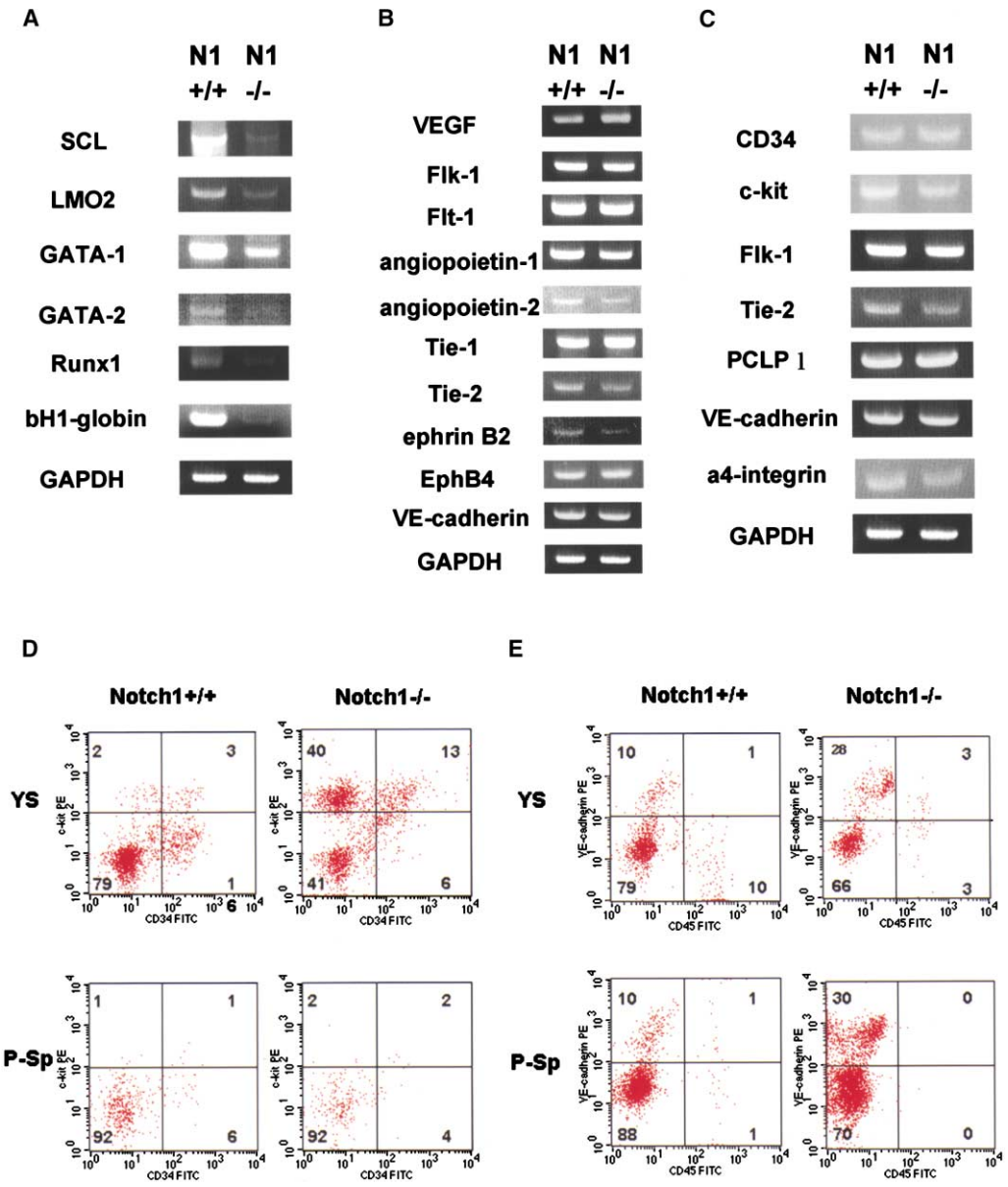

Figure 3. Expression of Hematopoietic Transcription Factors, Endothelial Growth Factors and Their Receptors, and Surface Markers of Hemangioblasts or Hemogenic Endothelial Cells

Expression of hematopoietic transcription factors (A) and endothelial growth factors and their receptors (B) was analyzed by semiquantitative RT-PCR to compare the level of expression between the P-Sp of *Notch1*$^{+/+}$ (N1+/+) and *Notch1*$^{-/-}$ (N1−/−) embryos.

Expression of surface markers of hemangioblasts or hemogenic endothelial cells was studied by RT-PCR (C) and FACS analysis (D and E). Representative data in three independent experiments are presented as FACS dot plots, and percentages of cells in each quadrant are indicated.

with the expression of these genes, although the deficiency of Notch1 may have an effect downstream of these gene products.

Cells Displaying the Phenotypes of Hemangioblast/Hemogenic Endothelial Cells Are Present in the *Notch1*$^{-/-}$ Embryo

We next studied whether hemangioblast/hemogenic endothelial cells might exist in the *Notch1*$^{-/-}$ embryo. Previously, several surface proteins were characterized as markers of these cells present in P-Sp/AGM or YS (Nishikawa et al., 1998; Hara et al., 1999; Ogawa et al., 1999; Sanchez et al., 1996; Marshall et al., 1999; Takakura et al., 1998). By a semiquantitative RT-PCR method, we compared the expression levels of these marker molecules. The results showed that there were no apparent differences in the expression levels between the control (*Notch1*$^{+/+}$) and *Notch1*$^{-/-}$ P-Sp (Figure 3C), suggesting that cells with characteristics of hemogenic endothelial cells do exist in the Notch1$^{-/-}$ embryo.

It was previously shown that HSC activity exists in the CD34(+)c-kit(+) or vascular endothelial (VE)-cadher-

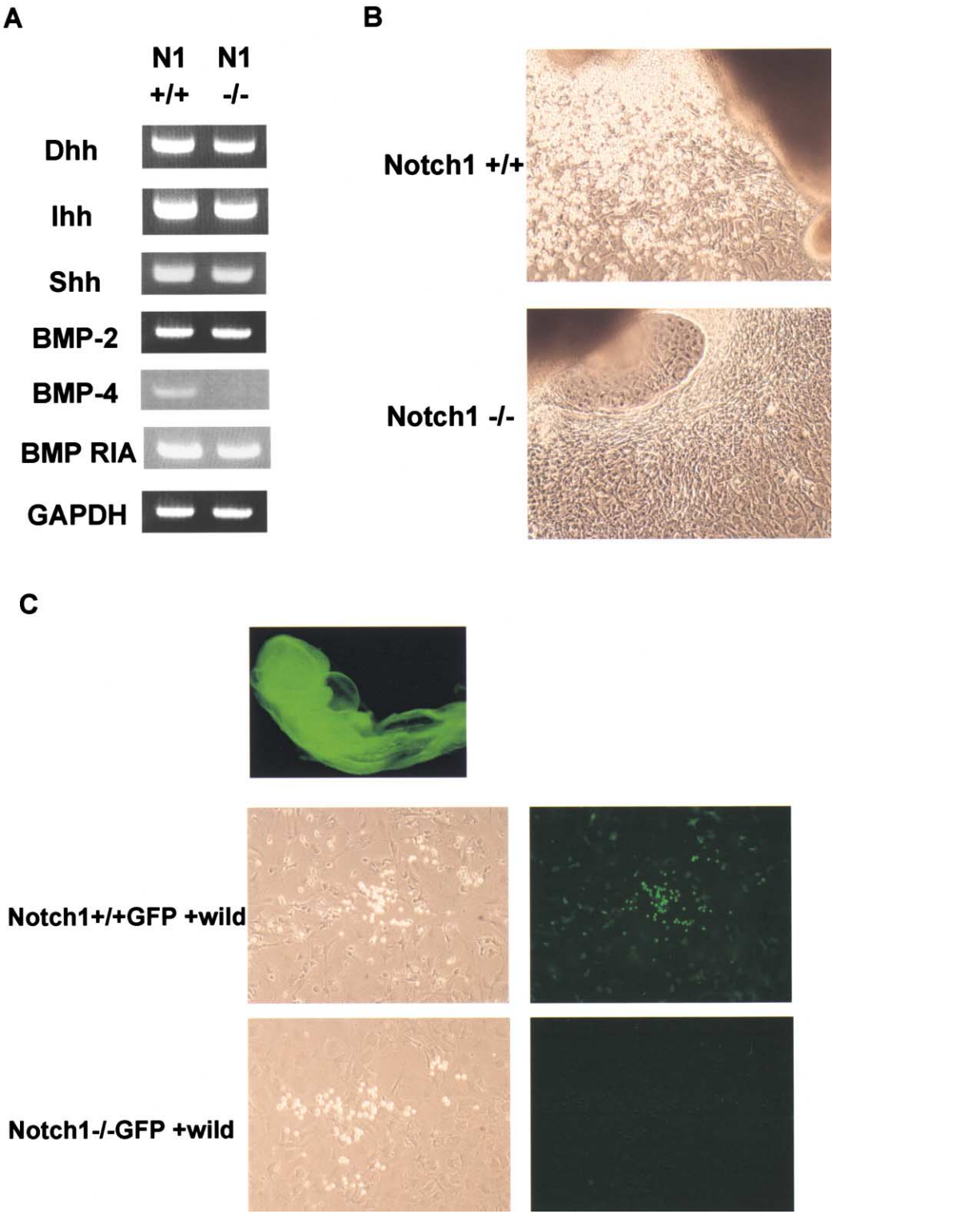

Figure 4. Effect of Microenvironmental Signals to the *Notch1*$^{-/-}$ P-Sp

Expression of the genes essential for the generation of hematopoietic cells was analyzed by RT-PCR (A). Dhh, Desert hedgehog; Ihh, Indian hedgehog; Shh, Sonic hedgehog; and BMP-RIA, BMP type IA receptor. (B) Addition of BMP-4 could not rescue the defect of development of hematopoietic cells in the *Notch1*$^{-/-}$ P-Sp at E 9.5 organ culture at day 7. Magnification, ×200. (C) Normal microenvironment of wild-type embryo could not rescue the hematopoietic deficiency. *Notch1*$^{-/-}$GFP(+) embryo was established (upper panel). Mixture of the dissociated *Notch1*$^{+/+}$GFP(-) P-Sp (E 9.5) (1×10^5 cells/ml) and *Notch1*$^{-/-}$GFP(+)P-Sp (1×10^5 cells/ml) were cultured on OP-9 cells (lower panel). No GFP(+) hematopoietic cells were detected at culture day 7.

in(+)CD45(-) cells in both YS and P-Sp (Yoder et al., 1997; Nishikawa et al., 1998). To identify the cells displaying surface expression of these markers, single cell suspensions of the YS and P-Sp of the *Notch1*$^{+/+}$ and *Notch1*$^{-/-}$ embryo were analyzed by flow cytometry. The ratios of CD34(+)c-kit(+) and VE-cadherin(+)CD45(-) cells were similar or increased in both YS and P-Sp of the *Notch1*$^{-/-}$ embryo compared to those of the *Notch1*$^{+/+}$ embryo (Figures 3D and 3E). As the total number of cells recovered from the individual YS or P-Sp of *Notch1*$^{-/-}$ embryo was reduced to approximately two-thirds (range, 0.5–1.0) of those of a Notch1$^{+/+}$ embryo, the total numbers of CD34(+)c-kit(+) and VE-cadherin(+)CD45(-) cells were similar in both Notch1$^{+/+}$ and Notch1$^{-/-}$ YS and P-Sp.

Table 1. In Vivo Hematopoietic Reconstitution by Transplanted Cells

Embryonic Tissue		Reconstituted/Transplanted		
		Notch1$^{+/+}$	Notch1$^{+/-}$	Notch1$^{-/-}$
YS	1 ee	2/3	4/6	0/3
	2 ee		3/3	0/2
	3 ee		1/1	0/1
P-Sp	1 ee	1/2	3/6	0/3
	2 ee		2/4	0/3
	3 ee		1/1	0/1

Cells from E 9.5 YS and P-Sp were injected into the conditioned newborn recipients. Transplanted cells were ranged from 1 to 3 embryo equivalents (ee). At 8–12 weeks after the transplant, donor cell contribution was analyzed using the Ly5.2 marker. More than 1% contribution was determined as positive.

Normal Microenvironment Cannot Rescue the Defect of Hematopoietic Development in the *Notch1*$^{-/-}$ Embryo Culture

To explore the possibility that the impaired hematopoiesis in the *Notch1*$^{-/-}$ mice was due to the lack of environmental cues essential for hematopoietic development, we first analyzed the expression of the candidate genes for these environmental molecules by a semiquantitative RT-PCR method. Previously, hedgehog and bone morphogenetic protein (BMP) families were shown to be engaged in this process (Dyer et al., 2001; Winnier et al., 1995; Kishimoto et al., 1997). Among them, BMP-4 was downregulated in *Notch1*$^{-/-}$ P-Sp (Figure 4A). To study whether lack of hemogenic potential of *Notch1*$^{-/-}$ P-Sp was due to impaired BMP-4 production, we added BMP-4 to the P-Sp organ culture. Addition of BMP-4, however, could not rescue the hemogenic deficiency of the *Notch1*$^{-/-}$ P-Sp (Figure 4B).

To further assess the involvement of other unidentified molecules, we cultured a mixture of cells prepared from wild-type and *Notch1*$^{-/-}$GFP(+) P-Sp. After a 7 day culture, hematopoietic cells appeared, but all of them were GFP(-), indicating that the normal environment did not rescue the defect in *Notch1*$^{-/-}$ P-Sp (Figure 4C). Therefore, it was further indicated that the defect of hematopoietic development in *Notch1*$^{-/-}$ embryo is attributed not to environmental effects but to the cell-autonomous effect in hemogenic endothelial cells.

HSC Activity Is Diminished in Both YS and P-Sp of the *Notch1*$^{-/-}$ Embryo

Using an irradiated adult mouse as a recipient, definitive HSC activity is first detectable in the AGM region of the embryo at day 10.5 (Medvinsky et al., 1993; Muller et al., 1994; Cumano et al., 1996; Medvinsky and Dzierzak, 1996). In contrast, when conditioned newborn mice are used as recipients, HSC activity can be detected as early as day 8 or 9 in both YS and P-Sp (Yoder et al., 1997). Because the *Notch1*$^{-/-}$ embryo dies at or soon after E10.5 and showed obvious developmental retardation after E9, we performed the latter method to determine whether the HSC activity exists in the *Notch1*$^{-/-}$ embryo.

We transplanted 1 to 3 embryo-equivalent (ee) cells from E9.5 YS and P-Sp of *Notch1*$^{+/+}$, *Notch1*$^{+/-}$, and *Notch1*$^{-/-}$ embryos (Ly 5.2) into a busulfan conditioned newborn recipient (Ly 5.1). At 2 months posttransplant, donor-derived Ly 5.2(+) cells could be detected in the peripheral blood of the recipients that received P-Sp cells from the *Notch1*$^{+/+}$ and *Notch1*$^{+/-}$ but not from the *Notch1*$^{-/-}$ embryos (Table 1), despite the fact that the YS cells from the *Notch1*$^{-/-}$ embryos had CFC activity as *Notch1*$^{+/+}$ and *Notch1*$^{+/-}$ embryos (Figure 2D). Thus, Notch1 is required for the generation of HSCs in both P-Sp and YS.

Notch1 Is Essential for the Step of Emergence of Hematopoietic Cells from Endothelial Cells

Notch1 has been shown to be essential for hematopoietic development. Although this could simply represent a secondary effect due to markedly impaired angiogenesis, it was hypothesized that hemangioblasts, the common progenitor for endothelial and primitive hematopoietic progenitors, might develop normally since vasculogenesis occurred normally and the CFC activity was maintained in YS. Our observation described above also suggested that the cells phenotypically characterized as hemogenic endothelial cells in P-Sp could develop normally in number.

To investigate the role of Notch signaling after the establishment of hemogenic endothelial cells, in the E9.5 wild-type P-Sp organ culture we used the γ-secretase inhibitor, which inhibits the release of the Notch intracellular domain and mimics the phenotype of conditional knockout mice in the fetal thymic organ culture (Doerfler et al., 2001; Hadland et al., 2001). Addition of the γ-secretase inhibitor significantly impaired the generation of hematopoietic cells similar to *Notch1*$^{-/-}$ P-Sp (Figure 5A). In the AGM explant culture at E10.5, however, when CD45(+) hematopoietic progenitors already exist, the presence of the γ-secretase inhibitor did not seem to affect the in vitro generation of hematopoietic cells (Figure 5A). To find the stage at which Notch signaling plays a key role, we separated VE-cadherin(+)CD45(-) hemogenic endothelial cells from E10.5 AGM and used them for the culture. In this experiment, addition of the γ-secretase inhibitor significantly impaired the generation of hematopoietic cells in the same manner as was observed in the experiment with the E9.5 P-Sp explant (data not shown). Therefore, the γ-secretase inhibitor impaired the generation of hematopoietic cells but did not affect their proliferation or maintenance. To rescue the γ-secretase inhibitor-induced repression

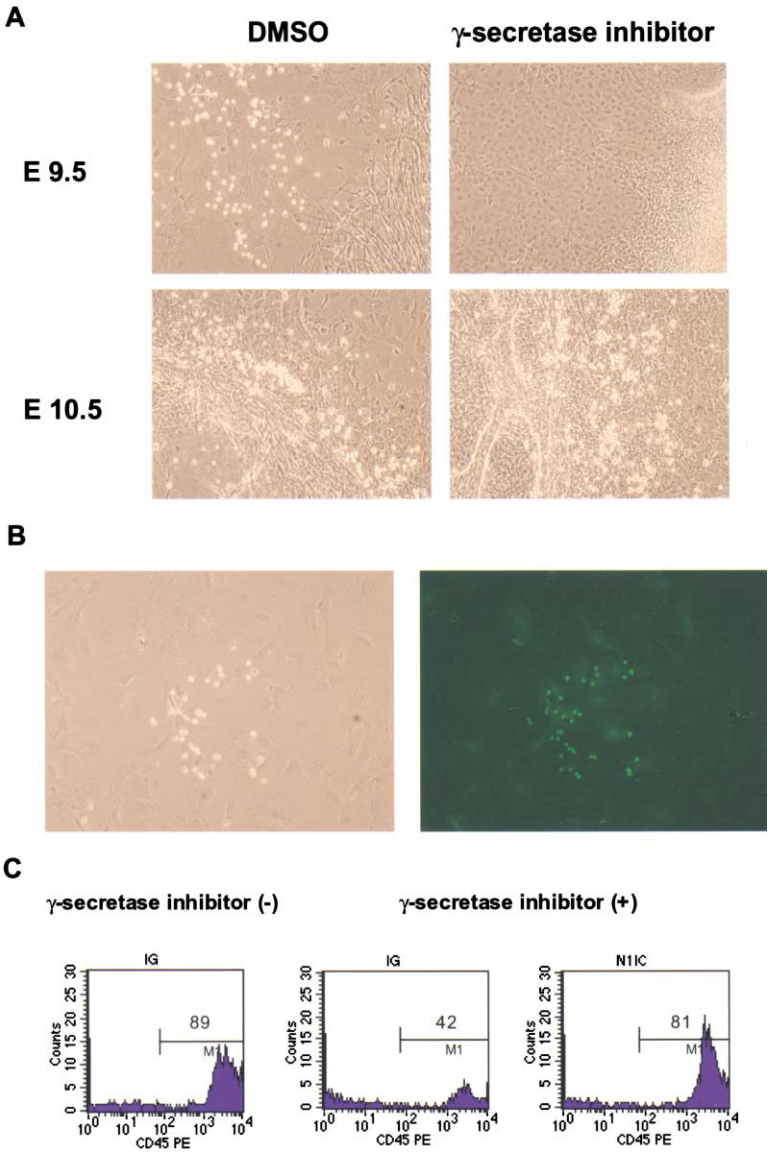

Figure 5. Addition of a γ-Secretase Inhibitor Mimics the *Notch1*$^{-/-}$ Phenotype and Notch1IC Rescues the γ-Secretase Inhibitor-Induced Block of Hematopoiesis

(A) The γ-secretase inhibitor significantly impaired the development of hematopoietic cells in the E 9.5 P-Sp organ culture of wild-type embryo but did not affect the proliferation or maintenance of hematopoietic cells from E 10.5 AGM. Development of hematopoietic cells was evaluated at culture day 7.

(B) Infection of retrovirus containing Notch1IC increased the hematopoietic cells in the presence of the γ-secretase inhibitor. Virus-infected cells showed GFP(+) at culture day 7.

(C) Histograms of the CD45-stained cells for the GFP-gated cell populations. Percentages of CD45(+) cells are indicated. Left and middle panels, infection with the control GFP virus without (left) or with (middle) the γ-secretase inhibitor. Right panel, infection with the N1IC vector with the γ-secretase inhibitor. Representative data in three independent experiments are presented.

of hematopoietic cell development, the cells from P-Sp were transduced with an intracellular portion of Notch1 (N1IC), which is constitutively active, using the pMYIR-ESGFP retrovirus vector that generates a bicistronic transcript encoding an internal ribosomal entry site (IRES) and GFP, and cultured in the presence of the γ-secretase inhibitor. The transduction efficiencies with control and N1IC vectors were almost equal (44% and 45%, respectively) (data not shown). In the control vector-transduced group, the addition of the γ-secretase inhibitor reduced the ratio of CD45(+) among the GFP(+) fraction at day 7 of culture. In contrast, even in the presence of the γ-secretase inhibitor, the N1IC vector-transduced GFP(+) cells gave rise to round cells indicating hematopoietic cells (Figure 5B), and most of the cells in the GFP(+) fraction expressed CD45 at a high level, which is similar to that in the absence of the γ-secretase inhibitor (Figure 5C). These results indicate that the reduction of Notch signaling blocked the generation of hematopoietic cells at E9.5 P-Sp, and that this inhibition of hematopoietic cell production was rescued by the introduction of constitutive active Notch1.

Notch2 Is Dispensable for the Development of Hematopoietic Cells

Notch2$^{-/-}$ mice develop normally until E9.5, and then around E11.5 lethal, widely distributed, massive cell death results (Hamada et al., 1999). We performed in vitro P-Sp organ culture from the *Notch2*$^{-/-}$ embryo (E9.5). No difference was observed in the hematopoietic cell development between the *Notch2*$^{-/-}$ and wild-type P-Sp (Figure 6A). The CFC activity of hematopoietic cells from the cultured P-Sp was also maintained (data not shown). The CFC activity in YS was not changed between the wild-type and *Notch2*$^{-/-}$ embryos (Figure 6B). Furthermore, the HSC activity was similar in both YS and P-Sp of the *Notch2*$^{-/-}$ embryo when investigated in the busulfan-conditioned newborn transplantation system (data not shown). These results indicate that Notch2

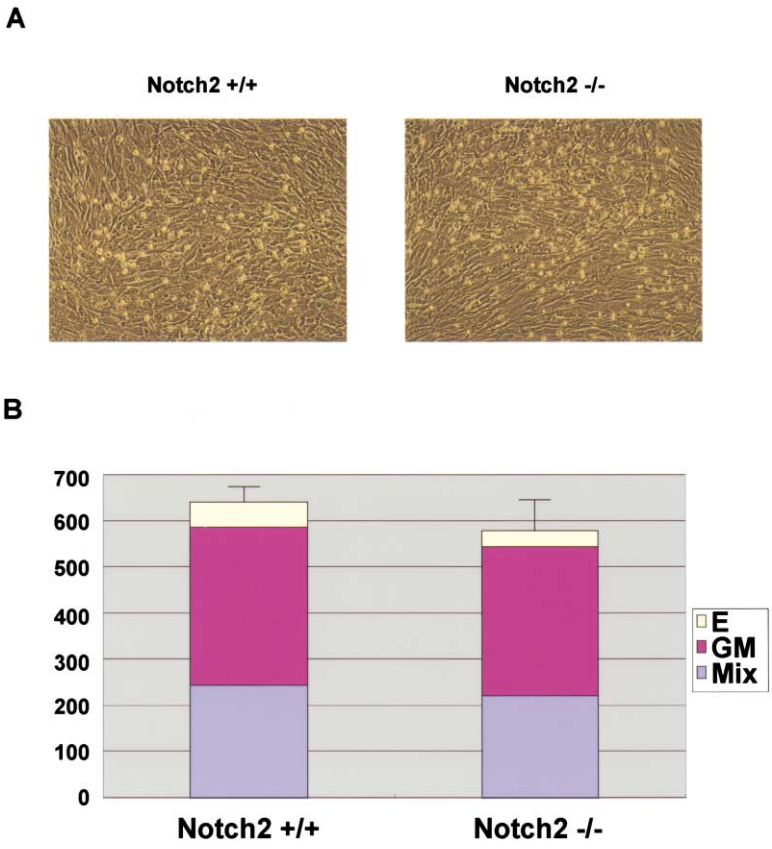

Figure 6. Normal Hematopoietic Development in the *Notch2*$^{-/-}$ Embryo

P-Sp organ culture from *Notch2*$^{+/+}$ and *Notch2*$^{-/-}$ embryos (A). Hematopoietic cells normally developed from the *Notch2*$^{-/-}$ embryo at culture day 7. Magnification, ×200. (B) CFC activity of YS-derived cells was almost the same in wild-type and *Notch2*−/− embryos. The results show the mean values of triplicate wells with standard deviations in one representative experiment from three independent experiments.

is not required for the embryonic development of hematopoietic cells.

Discussion

In this study, we investigated the role of Notch signaling in the development of hematopoiesis. We have shown that the expression patterns of Notch1, Notch2, and Notch4 are distinct between endothelial cells and hematopoietic cells. Among them, Notch1 is expressed in both CD45(-) endothelial cells and CD45(+) hematopoietic cells (Figure 1). Since several genes essential for hematopoiesis (Flk-1, SCL, Lmo2, GATA-2, Runx1, etc.) are expressed in both endothelial cells and hematopoietic cells, we hypothesized that Notch1 is also a regulator for hematopoiesis.

The Role of Notch1 in the Development of Definitive Hematopoietic Cells

Hematopoietic cells and vascular cells share common origins and appear to be closely related in the developmental process. In YS, several reports support the existence of bipotenital hemangioblasts (Pardanaud et al., 1989; Choi et al., 1998; Jaffredo et al., 1998). In vitro differentiation of embryonic stem (ES) cells shows their presence, although short-lived. In the embryo proper, HSCs appear to arise from a subset of the vascular compartment (hemogenic endothelial cells) but only at limited sites such as the ventral wall of the dorsal aorta and proximal umbilical and vitelline arteries (Pardanaud et al., 1989; Jaffredo et al., 1998; Tavian et al., 1996; De Bruijn et al., 2000). VE-cadherin is described as being expressed in endothelial cells. VE-cadherin(+)CD45(-) cells sorted from YS or from the caudal half of the embryo containing P-Sp/AGM have been shown to differentiate in vitro into endothelial and lymphohematopoietic progenies (Nishikawa et al., 1998).

In the *Notch1*$^{-/-}$ embryo, hemangioblasts appear to be intact because neither vasculogenesis in the YS and embryo proper nor primitive hematopoiesis are impaired, as described here. To date, several investigators have independently defined hemogenic endothelial cell populations by different sets of cell surface markers such as c-kit(+)CD34(+) (Sanchez et al., 1996), Flk-1(+) (Marshall et al., 1999), Tie-2(+) (Takakura et al., 1998), VE-cadherin(+)CD45(-) (Nishikawa et al., 1998), PCLP-1(+)CD45(-) (Hara et al., 1999), and α4-integrin(+) (Ogawa et al., 1999). In our experiment, c-kit(+)CD34(+) and VE-cadherin(+)CD45(-) cells are found in both P-Sp and YS of the *Notch1*$^{-/-}$ embryo as well as the *Notch1*$^{+/+}$ embryo. Surprisingly, the ratios of these cell populations were not decreased in the *Notch1*$^{-/-}$ YS (Figures 3D and 3E). Notably, an unexplained increase of the c-kit(+)CD34(-) population in the *Notch1*$^{-/-}$ YS was demonstrated (Figure 3D), although its significance is unknown. Nevertheless, specification into the hemogenic endothelial cells recognized by these surface markers is intact in the *Notch1*$^{-/-}$ embryo. It is likely to be capacitating endothelial cells for hematogenicity that is defective in the *Notch1*$^{-/-}$ embryo.

Recently, it was reported that Notch pathway molecules are essential for the maintenance, but not for the

generation, of mammalian neural stem cells (Hitoshi et al., 2002). This could raise the possibility that Notch1 functions in the maintenance or expansion of HSCs, rather than their generation. However, both currently described and previously reported observations conflict with this possibility. For example, we have shown that the addition of a γ-secretase inhibitor significantly impaired the generation of hematopoietic cells from the explanted wild-type E9.5 P-Sp (Figure 5A) and from E10.5 AGM-derived VE-cadherin(+)CD45(-) cells (data not shown), but not from the explanted wild-type E10.5 AGM, where CD45(+) hematopoietic cells already exist. Furthermore, in a previous report using a conditional gene targeting system in which *Notch1* was disrupted after birth, *Notch1*-deficient bone marrow cells contributed normally to all hematopoietic lineages other than the T cell lineage in a lethally irradiated adult recipient (Radtke et al., 1999). These results indicate that Notch1 acts at the step of the generation but not the expansion or maintenance of hematopoietic stem cells.

The origin of HSCs is still controversial. An avian (Dieterlen-Lievre, 1975) and amphibian (Turpen et al., 1981) chimera systems showed that circulating YS-derived hematopoietic cells are rapidly replaced by those of intraembryonic origin. More recently, several lines of evidence have shown that in mice, HSCs, which can reconstitute to a lethally irradiated adult recipient, exist only in the AGM region in the embryo proper at E 10.5 while YS cells show comparable activity only after E11 (Medvinsky and Dzierzak, 1996; Muller et al. 1994). Both YS and P-Sp cells from E9 embryos, however, provide multilineage reconstitution in conditioned newborn recipients and in secondary adult recipients in mice (Yoder et al., 1997). Furthermore, high proliferative potential colony-forming cells (HPP-CFCs) are found exclusively within the YS at E8.25 (Palis et al., 1999). Therefore, one possibility is that these YS-derived newborn reconstitutable cells seed in the AGM region and acquire the potential as definitive HSCs. The other possibility is that definitive HSCs are generated de novo in the AGM regions. These two possibilities are not necessarily exclusive of each other. Given the reports that the B lymphopoietic progenitors in YS at E9.5 are enriched in the VE-cadherin(+)CD45(-) population (Nishikawa et al., 1998) and that PCLP-1(+)CD45(-) cells but not CD45(+) cells from AGM region are transplantable (Hara et al., 1999), the major reconstitutable cells in both YS and P-Sp/AGM are likely to be hemogenic endothelial cells. Taking this notion together with our findings that CFC activity is maintained in E9.5 YS while the newborn reconstitutable activity is lost in the *Notch1*$^{-/-}$ embryo, we prefer the hypothesis that definitive HSCs are generated de novo in the AGM region in the physiological condition.

The microenvironment in AGM is thought to be essential for HSC's development (Matsuoka et al., 2001; Marshall et al., 1999). Several molecules such as hedgehog (Dyer et al., 2001) and BMP family proteins (Winnier et al., 1995; Kishimoto et al., 1997) are reported to be involved in the development of hematopoiesis. Among them, only BMP-4 is downregulated in the P-Sp region of the *Notch1*$^{-/-}$ embryo. This is consistent with the recent report that overexpression of Delta1, one of the Notch ligands, induced BMP-4 expression (Endo et al., 2002). BMP-4 is essential for the in vitro differentiation of ES cells into hematopoietic lineages (Nakayama et al., 2000; Li et al., 2001) and is expressed at high levels with a striking polarity in a region of densely packed cells underlying intra-aortic hematopoietic clusters in the AGM regions (Marshall et al., 2000). These findings suggest that BMP-4 might be a major effector downstream of the Notch signaling. The addition of BMP-4 to the *Notch1*$^{-/-}$ P-Sp organ culture, however, could not rescue the development of hematopoietic cells (Figure 4B). This implies that downregulation of BMP-4 expression is not the sole reason for the defect in definitive hematopoiesis. The expression of other TGF-β family molecules is intact in the *Notch1*$^{-/-}$ mice (data not shown).

Targeted disruption of various transcription factors indicates that SCL/tal-1 (Porcher et al., 1996), Lmo-2 (Yamada et al., 1998), and GATA-1 (Pevny et al., 1991) are required for both primitive and definitive hematopoiesis and that Runx1 (Okuda et al., 1996) and GATA-2 (Tsai et al., 1994) are required mainly for definitive hematopoiesis, although primitive hematopoiesis may also be affected to some extent. It is shown in this paper that all of these transcription factors are downregulated in the *Notch1*$^{-/-}$ P-Sp, indicating that Notch1 at least partially controls and possibly uses these molecules in the downstream signaling machineries in definitive, although not primitive, hematopoiesis. Recently, it was reported that Runx and Smad proteins function together in gene regulation (Zaidi et al., 2002). In the AGM region, Runx1 and Smads activated by BMP-4 may constitute the active form of a transcriptional complex. GATA-2 is expressed in the endothelial cells in the P-Sp/AGM region (Minegishi et al., 1999) and is also under the control of BMP-4 (Maeno et al., 1996). We previously reported that Notch1 maintains the expression of GATA-2 (Kumano et al., 2001). All these results support the potential relevance between Notch1 and the hematopoietic transcription factors described above, although more precise molecular mechanisms need to be clarified in the future.

The Role of Notch1 in Angiogenesis

Notch signaling plays an important role during vascular development in mice (Gridley, 2001). Both Notch1 and Notch1/Notch4 double mutant embryos displayed severe defects in angiogenic vascular remodeling (Krebs et al., 2000). Using the P-Sp organ culture system, we have currently shown impaired angiogenesis in the *Notch1*$^{-/-}$ embryo. Recently, it was reported using the same system that Runx1 is involved in vascular remodeling through the regulation of angiopoietin-1 production by hematopoietic cells (Takakura et al., 2000). However, addition of the P-Sp-derived hematopoietic cells to the *Notch1*$^{-/-}$ P-Sp organ culture cannot rescue angiogenesis (data not shown) unlike the observation that hematopoietic cells rescue angiogenesis in the Runx1$^{-/-}$ P-Sp organ culture. Moreover, the angiogenic defect appears to be more severe in the *Notch1*$^{-/-}$ embryo than in the *Runx1*$^{-/-}$ embryo. Nevertheless, the expression levels of several angiogenic cytokines and their receptors were unaffected in the *Notch1*$^{-/-}$ embryo (Figure 3B). The reason angiogenesis is impaired in the *Notch1*$^{-/-}$ embryo remains unknown.

Notch2 Is Dispensable for the Development of Hematopoietic Cells

Since both Notch1 and Notch2 are expressed in HSCs (Bigas et al., 1998), and Notch1 and Notch2 knockout mice die at a similar midgestation stage, Notch2-deficient mice may have a defect in HSC function. However, it is clearly shown here that Notch2 is unnecessary for the hemogenic capacity, which is proven by the in vitro and in vivo hematopoietic cell production experiments (Figure 7; data not shown). We can thus conclude that Notch1 and Notch2 function differently in the context of the development of hematopoiesis.

Experimental Procedures

Generation of Mice and Embryos

C57BL6 mice and *Notch1* mutant mice (Conlon et al., 1995) were purchased from Japan SLC and Jackson laboratory. *Notch2* mutant mice were previously generated (Hamada et al., 1999). Transgenic mice with GFP were a gift from Dr. M. Okabe (Osaka University, Osaka, Japan) (Okabe et al., 1997). *Notch1*$^{-/-}$ GFP (+) embryos were obtained from *Notch1*$^{+/-}$ GFP(+) mice that were generated by crossing *Notch1*$^{+/-}$ and GFP mice. The time at midday (12:00) was taken to be E0.5 for the plugged mice.

Cell Preparation

Isolated P-Sp/AGM region and YS of E9.5–11.5 were dissociated by incubation with 250 U/ml dispase (Godo Shusei) for 30 min at 37°C and cell dissociation buffer (Gibco BRL) for 5 min followed by vigorous pipetting.

In Vitro P-Sp Organ Culture and P-Sp/AGM Cell Culture

P-Sp organ culture was performed as described previously (Takakura et al., 1998) with a minor modification. In brief, P-Sp explants from E9.5 embryo were seeded on OP-9 stromal cells in RPMI1640 (Sigma) with 10% fetal calf serum (FCS) supplemented with 50 ng/ml stem cell factor (SCF) and 5 ng/ml interleukin3 (IL3) (gifts from Kirin Brewery, Takasaki, Japan). BMP-4 (R&D) was added to the P-Sp organ culture of *Notch1*$^{-/-}$ embryo at 100 ng/ml.

Single cell suspensions (1×10^5 cells/ml in the 12-well plate) from P-Sp/AGM were also seeded on OP-9 cells in the condition described for the organ culture. For mixed culture for *Notch1*$^{+/+}$GFP(-) and *Notch1*$^{-/-}$GFP(+) P-Sp, the single cell culture system was used after mixing 1×10^5 cells from each P-Sp in the 12-well plate.

γ-secretase inhibitor II (CALBIOCHEM) was added to the P-Sp/AGM organ culture and single cell suspension culture at 50 μM.

Immunostaining

Immunostaining was performed as described previously (Yamaguchi et al., 2002) with a minor modification. In brief, cells from P-Sp and AGM or explants from P-Sp were cultured on OP-9 stromal cells (Takakura et al., 1998). The cultured samples were fixed with -20°C methanol for 15 min for immunofluorescence staining or with 2% paraformaldehyde for 30 min at room temperature for peroxidase staining. Samples were permeabilized with phosphate-buffered saline (PBS) containing 0.1% NP-40 for 10 min and incubated for 40 min with 5% BSA in PBS for blocking. The cell layer was then covered with goat antibodies against Notch1 and Notch2 (Santa Cruz Biotechnology), a rabbit antibody against Notch4 (a gift from Y. Shirayoshi), and rat antibodies against CD45, CD34, CD31 (PECAM-1), and Flk1 (Pharmingen) and incubated for 60 min. The washed cell layer was incubated with a fluorescein isothiocyanate (FITC)-conjugated anti-goat or anti-rabbit IgG (ICN pharmaceuticals) and Cy-3-conjugated anti-rat IgG (Jackson Immuno Research Laboratories) secondary antibodies for 45 min. For peroxidase staining of the samples from P-Sp organ culture, the samples were fixed and blocked for endogenous peroxidase activity with 4% H_2O_2 in PBS. Then, they were incubated with anti-CD31 for 60 min, rinsed, incubated with secondary antibody conjugated with horseradish peroxidase, rinsed, and stained with DAB for 10 min. For endothelial cell labeling, 10 μg/ml acetylated low-density lipoprotein labeled with DiI-Ac-LDL (Biomedical Technologies) was added for 6 hr at 37°C.

Immunostaining of sections from frozen embryo (E11.5) was performed as previously described (Sata et al., 2002). In brief, the frozen sections were first stained with antibodies (anti-Notch1, anti-CD31) followed by incubation with FITC or Cy-3-conjugated secondary antibodies. The sections were mounted with the ProLong Antifade Kit (Molecular Probes) and observed under a confocal microscope (FLUOVIEW FV300, Olympus).

CFC Assay

Fresh total cells from YS or cells that were recovered by pipetting alone from the P-Sp organ culture were used for CFC assay. Cells from 1 embryo equivalent P-Sp organ culture or 1×10^5 cells from YS were seeded in 3 ml of the methylcellulose (Stem Cell Technologies) supplemented with 100 ng/ml SCF, 10 ng/ml IL3, and 2 U/ml erythropoietin (EPO) (gift from Kirin Brewery, Takasaki, Japan). Colony types were scored at culture day 7 by morphological appearance and by Wright-Giemsa staining of each colony.

RT-PCR Analysis

The procedures for semiquantitative RT-PCR analysis have been described elsewhere (Kumano et al., 2001). Sequences of specific primers used in RT-PCR and PCR conditions can be requested. YS or P-Sp with the same genotype were pooled to prepare the total RNA.

Flow Cytometry Analysis

Flow cytometry analysis was performed in a FACScalibur with the Cellquest program (Becton Dickinson). For surface staining, cell suspensions from individual P-Sp or YS were incubated on ice in the presence of various mixtures of labeled mAb. The ly5 alleles were characterized using biotinylated, phycoerythrin (PE)-, or fluorescein isothiocyanate (FITC)-conjugated antibodies 104.2 (anti-Ly5.2) or A20.17 (anti-Ly5.1). PE-conjugated anti-Gr-1, anti-Mac-1, anti-CD3, and anti-B220 antibodies were used to examine lineage contribution. FITC-conjugated anti-CD34, PE-conjugated anti-c-kit, and purified anti-VE-cadherin antibodies were used for the characterization of YS and P-Sp cells. All antibodies were purchased from Pharmingen.

Transplantation Assay

Transplantation of cells into busulfan-treated neonatal mice was performed as previously described (Yoder et al., 1997) with a slight modification. In brief, pregnant mice were intraperitonially injected with busulfan (Sigma) at 18.75 μg/g on pregnancy day 17 and 18. Within 24–48 hr after birth, cells from E 9.5 YS or P-Sp ranging from 1 to 3 embryo equivalents were prepared in 25 μl PBS and injected into the neonatal liver.

Peripheral blood (PB) was collected from transplanted mice 8–12 weeks after the transplant. Red blood cells were lysed by Red Blood Cell Lysing Buffer (Sigma). Donor-derived cells were detected by FACS analysis using the ly5.2 marker and lineage contribution was evaluated using Gr-1 and Mac-1 (myeloid), CD3 (T cell), and B220 (B cell).

Retroviral Transduction

Notch1IC (N1IC) cDNA (Kumano et al., 2001) was subcloned into the retrovirus vector pMY/IRES-EGFP (pMY/IG) (Kitamura et al., in press), a gift from T. Kitamura (IMSUT, Tokyo). The resulting pMY/N1IC-IG and mock pMY/IG were transfected into ψMP34 packaging cells (a gift from Wakunaga Pharm., Hiroshima, Japan), which were single cell-sorted for GFP with the FACS Vantage (Becton Dickinson). Retrovirus transduction to the P-Sp cells was performed as described previously (Mukouyama et al., 2000). In brief, single cell suspensions (1×10^5 cells/ml in the 12-well plate) were incubated with viral supernatant, 10 μg/ml Polybrene (Sigma), and the set of cytokine on OP-9 cells. After 48 hr of incubation, virus-containing medium was replaced by standard culture medium. Retrovirus-infected cells were evaluated by the expression of GFP.

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